Antibacterial response of surface polarized and compositionally modified biocompatible

$Na_xK_{1-x}NbO_3$ (x = 0.2 – 0.8) ceramics

In this chapter, the antibacterial response of polarized piezoelectric $Na_xK_{1-x}NbO_3$ (x = 0.2 to 0.8) bioceramics *is discussed. The dense bioceramic samples were polarized via corona poling at polarizing temperature and voltage of 500 °C and 25 kV, respectively, for 30 min. Thereafter, the effect of surface polarization on viability and adhesion of S. aureus and E. coli bacteria on piezoelectric $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) as well as hydroxyapatite (HA, control) samples was analyzed, quantitatively as well as qualitatively. Furthermore, various assays associated with enzymatic activities were also performed to examine the effect of polarization and compositional variation on the generation of reactive oxygen species (ROS).*

5.1. Quantitative antibacterial analyses or MTT assay

Quantitative assessment of antibacterial response on the surface of non-polarized and polarized $Na_xK_{1-x}NbO_3$ and HA samples for *S. aureus* and *E. coli* bacteria is presented in Fig. 5.1 Statistical analyses revealed that all non-polarized and polarized $Na_xK_{1-x}NbO_3$ samples show significantly higher antibacterial response as compared to non-polarized HA. The population density of *S. aureus* and *E. coli* bacteria on non-polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples are reduced by (18, 10, 15 %) and (20, 17, 22 %), respectively, as compared to non-polarized HA. Antibacterial response of NKN samples is further improved by polarization as the positively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples reduced the viability of *S. aureus* bacteria by (41, 29, 50 %) and (29, 21, 41 %) as compared to the surfaces of non-polarized HA and non-polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples, respectively. Whereas, negatively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and

0.8) samples reduced the viability of *S. aureus* bacteria by (28, 20, 30 %) and (12, 11, 17 %) than the surfaces of non-polarized HA and non-polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples, respectively. However, in case of *E. coli* bacteria, the negatively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) demonstrate the reduction in bacterial population by (49, 37, 52 %) and (37, 24, 39 %) as compared to non-polarized surfaces of HA and $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples, respectively. On the other hand, the viability of similar bacteria is reduced on the positively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples by (31, 24, 45 %) and (14, 09, 30 %) as compared to non-polarized surfaces of HA and non-polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples, respectively. Among NKN samples, *S. aureus* bacterial adhesion is comparatively lower on positively polarized sodium rich NKN i.e., $Na_{0.8}K_{0.2}NbO_3$ (50 % of non-polarized HA) and potassium rich NKN i.e., $Na_{0.2}K_{0.8}NbO_3$ (59 % of non-polarized HA) samples than $Na_{0.5}K_{0.5}NbO_3$ (71 % of non-polarized HA) samples. Similar trends are observed in case of *E. coli* bacteria, where bacterial population is lowered on the negatively polarized sodium rich NKN i.e., $Na_{0.8}K_{0.2}NbO_3$ (48 % of non-polarized HA) and potassium rich NKN i.e., $Na_{0.2}K_{0.8}NbO_3$ (51 % of non-polarized HA) samples as compared to $Na_{0.5}K_{0.5}NbO_3$ (63 % of non-polarized HA) samples.
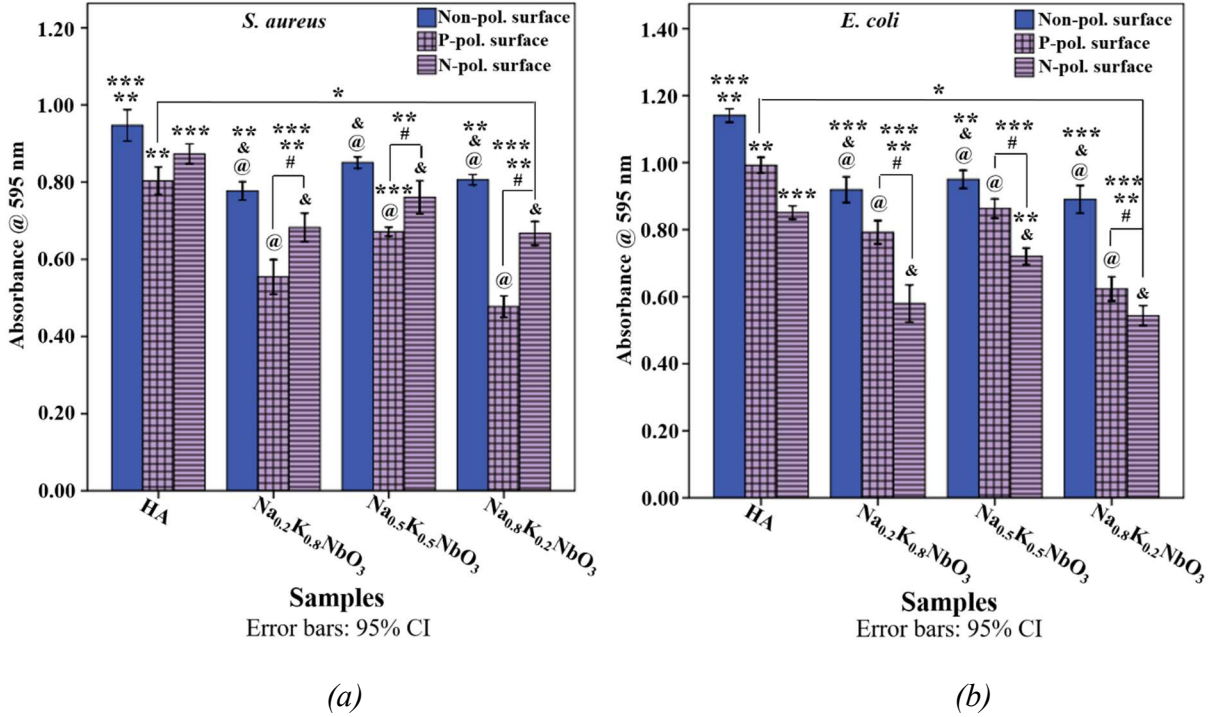
Fig. 5.1. Quantitative analyses of antibacterial response on the non-polarized and polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) and HA samples for (a) S. aureus and (b) E. coli bacteria. Asterisk (*), (**) and (***) mark represents the statistically significant difference at $p \leq 0.05$ in mean optical density among all the samples with respect to non-polarized, negatively polarized and positively polarized HA, respectively. The symbols (#), (@) and (&) represent the significant difference at $p \leq 0.05$ in mean optical density among the $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples with respect to their corresponding non-polarized, negatively polarized and positively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples.

Fig. 5.2 (a) and (b) illustrates the comparative difference among antibacterial ratio % (calculated from eqn. 3.5, chapter 3) for (a) *S. aureus* and (b) *E. coli* bacteria on non-polarized and polarized $Na_xK_{1-x}NbO_3$ and HA samples. The antibacterial ratio, measured on the surfaces of non-polarized, negatively and positively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples for *S. aureus* bacteria are (23.8, 16.6, 20.9 %), (33.1, 25.4, 34.6 %), (45.6,

34.2, 53.2 %), respectively, which was just 7.1 %, 14.4 % and 21.2 % on the surfaces of non-polarized, negatively and positively polarized HA samples, respectively. For *E. coli* bacteria, the antibacterial ratio on the non-polarized, negatively and positively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples are calculated to be (29.3, 26.9, 31.5 %), (55.4, 44.6, 58.2 %), (39.1, 33.6, 52.1 %), respectively. However, it was 12.2 %, 34.5 % and 23.7 % for the surfaces of non-polarized, negatively and positively polarized HA samples, respectively. The statistical analyses of the above mentioned results revealed that the non-polarized, negatively and positively polarized $Na_xK_{1-x}NbO_3$ samples show significantly higher antibacterial ratio as compared to non-polarized, negatively and positively polarized HA, respectively, for both the bacteria. As far as the antibacterial ratio among three polarized NKN samples is concerned, the antibacterial ratio is observed to be higher with both positively polarized sodium rich NKN i.e., $Na_{0.8}K_{0.2}NbO_3$ samples (7.4 times of non-pol. HA) and potassium rich NKN i.e., $Na_{0.2}K_{0.8}NbO_3$ (6.4 times of non-pol. HA) samples than the positively polarized $Na_{0.5}K_{0.5}NbO_3$ (4.8 times of non-pol. HA) samples for *S. aureus* bacteria. Similarly, negatively polarized $Na_{0.8}K_{0.2}NbO_3$ (4.8 times of non-pol. HA) and $Na_{0.2}K_{0.8}NbO_3$ (4.6 times of non-pol. HA) samples show comparatively higher antibacterial ratio for *S. aureus* bacteria than negatively polarized $Na_{0.5}K_{0.5}NbO_3$ (3.6 times of non-pol. HA) samples. In case of *E. coli* bacterial culture, the antibacterial ratios are higher for both, negatively polarized sodium rich NKN i.e., $Na_{0.8}K_{0.2}NbO_3$ (4.7 times of non-pol. HA) and potassium rich NKN i.e., $Na_{0.2}K_{0.8}NbO_3$ (4.5 times of non-pol HA) samples as compared to negatively polarized $Na_{0.5}K_{0.5}NbO_3$ (3.6 times of non-pol. HA) samples. Similarly, for positively polarized samples, sodium rich NKN i.e., $Na_{0.8}K_{0.2}NbO_3$ (4.2 times of non-pol. HA) and potassium rich NKN i.e., $Na_{0.2}K_{0.8}NbO_3$ (3.2 times of non-pol. HA) samples show

comparatively higher antibacterial ratios than positively polarized $Na_{0.5}K_{0.5}NbO_3$ (2.7 times of non-pol. HA) samples.
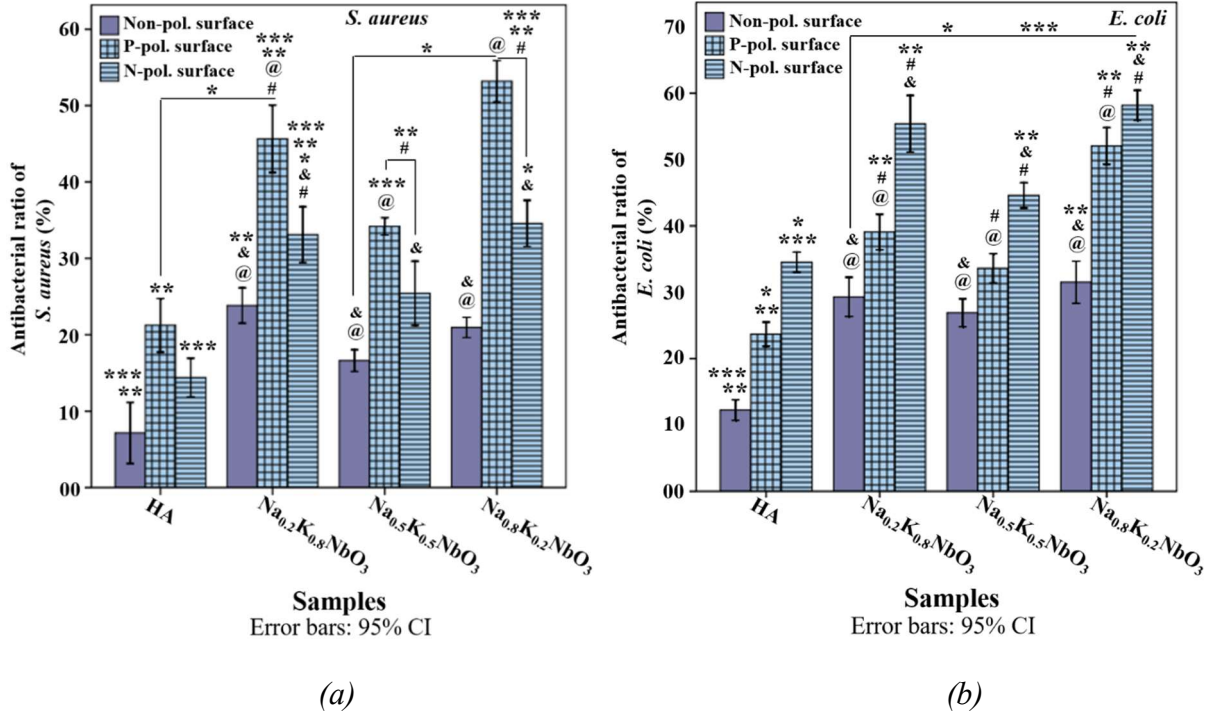

(a)   (b)

Fig. 5.2. Antibacterial ratio % (calculated from eqn. 3.5, chapter 3) for the non-polarized and polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) and HA samples with (a) S. aureus and (b) E. coli bacteria. Asterisk (*), (**) and (***) mark represents the statistically significant difference at p ≤ 0.05 in the mean value of antibacterial ratio among all the samples with respect to non-polarized, negatively polarized and positively polarized HA, respectively. The symbols (#), (@) and (&) represent the significant difference at p ≤ 0.05 in the mean value of antibacterial ratio among all the $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples with respect to their corresponding non-polarized, negatively polarized and positively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples.

5.2. Qualitative analyses

5.2.1. Live/ dead assay

Fig. 5.3 (a) and (b) represents the fluorescent images of live and dead bacterial cells, adhered on non-polarized and polarized $Na_xK_{1-x}NbO_3$ and HA samples. It is clearly observed that all the non-polarized and polarized $Na_xK_{1-x}NbO_3$ samples show less population of live bacteria than that of non-polarized HA sample for both, *S. aureus* and *E. coli* bacterial cells. In addition, the populations of live bacteria are the lowest on positively and negatively polarized $Na_xK_{1-x}NbO_3$ and HA samples for *S. aureus* [Fig. 5.3 (a)] and *E. coli* [Fig. 5.3 (b)] bacteria, respectively. A large amount of dead *S. aureus* and *E. coli* bacterial populations can be seen on the positively polarized $Na_xK_{1-x}NbO_3$ and HA samples, which is highest on the sodium and potassium rich NKN i.e., $Na_{0.8}K_{0.2}NbO_3$ and $Na_{0.2}K_{0.8}NbO_3$ samples.
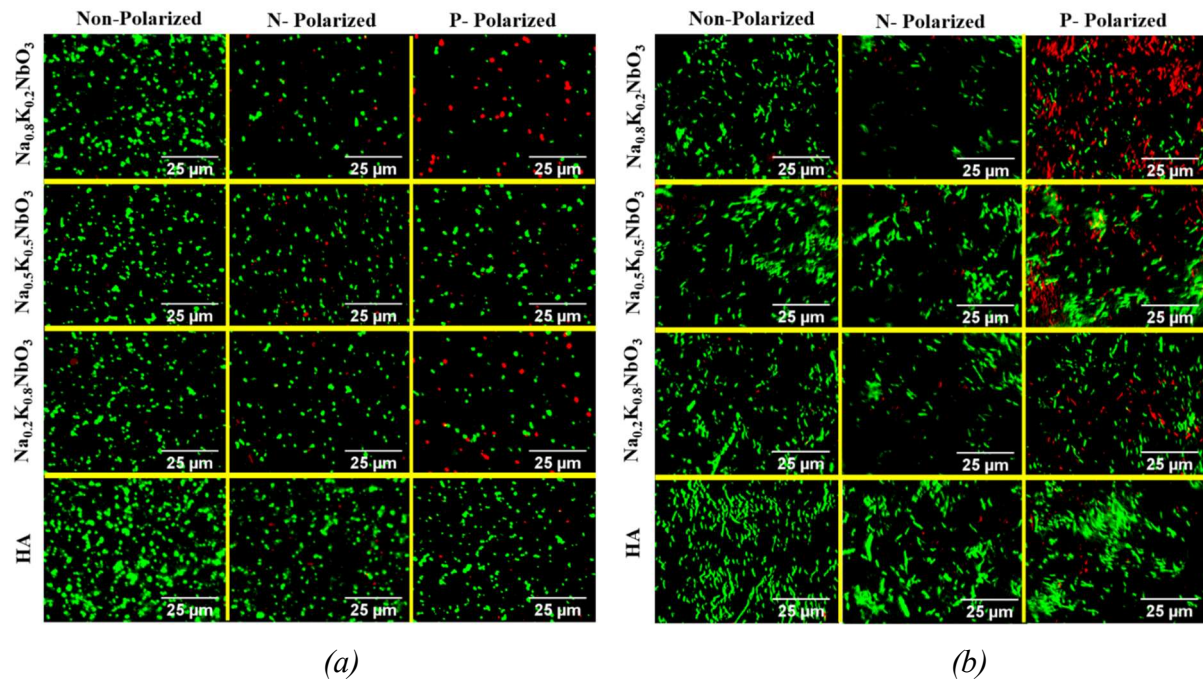

(a)   (b)

Fig. 5.3. Florescence microscopy images representing the live and dead strain of (a) S. aureus and (b) E. coli bacteria, exposed on non-polarized and polarized $Na_xK_{1-x}NbO_3$ (x =

0.2 - 0.8) and HA samples after 10 h of incubation in growth medium. Live and dead bacteria are strained green and red, respectively. Scale bar = 25 μm.

5.2.2. Disk diffusion method of colony counting

Fig. 5.4 represents the colonies along with their corresponding antibacterial ratio for *S. aureus* and *E. coli* bacteria, while cultured on non-polarized and polarized $Na_xK_{1-x}NbO_3$ as well as HA samples. The colony counting and corresponding antibacterial ratio results reveal that all the examined NKN (non-polarized and polarized) samples show significantly reduced bacterial colonies as compared to non-polarized and polarized HA samples, respectively. It can be clearly seen that, the population of diffused bacterial colonies are the lowest on positively and negatively polarized $Na_xK_{1-x}NbO_3$ samples for *S. aureus* and *E. coli* bacteria, respectively [5.4 (A) and (C)]. For *S. aureus* bacteria, the number of colonies on the surfaces of non-polarized, negatively and positively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples are (286, 328, 243), (185, 185, 118), (65, 109, 47), respectively. However, it was 493, 417 and 364 on the surfaces of non-polarized, negatively and positively polarized HA samples, respectively. In case of *E. coli* bacteria, the number of colonies on the non-polarized, negatively and positively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples were calculated as (375, 411, 259), (108, 134, 71), (207, 222, 205), respectively. However, it was 511, 293 and 426 on the surfaces of non-polarized, negatively and positively polarized HA samples, respectively. The antibacterial performance of non-polarized and polarized $Na_xK_{1-x}NbO_3$ samples was further evaluated by calculating antibacterial ratio (calculated from eqn. 3.6, chapter 3) against bacterial colonies [5.4 (B) and (D)]. Among all the samples, the positively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples show comparatively higher antibacterial ratio than their respective negatively polarized and

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non-polarized counterparts, for *S. aureus* bacteria. In addition, the antibacterial ratio are higher on the positively polarized sodium ($Na_{0.8}K_{0.2}NbO_3$) and potassium ($Na_{0.2}K_{0.8}NbO_3$) rich compositions as compared to positively polarized $Na_{0.5}K_{0.5}NbO_3$ samples. In case of *E. coli* bacteria, the antibacterial ratios are highest on the surfaces of negatively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5 and 0.8) samples than their respective positively polarized and non-polarized NKN and HA samples. Whereas, the negatively polarized sodium and potassium rich compositions of NKN show comparatively higher antibacterial ratio than negatively polarized $Na_{0.5}K_{0.5}NbO_3$ samples. Therefore, disk diffusion assay further corroborates that polarized surfaces inhibit growth of bacteria, which is higher on the sodium ($Na_{0.8}K_{0.2}NbO_3$) and potassium ($Na_{0.2}K_{0.8}NbO_3$) rich compositions of NKN.
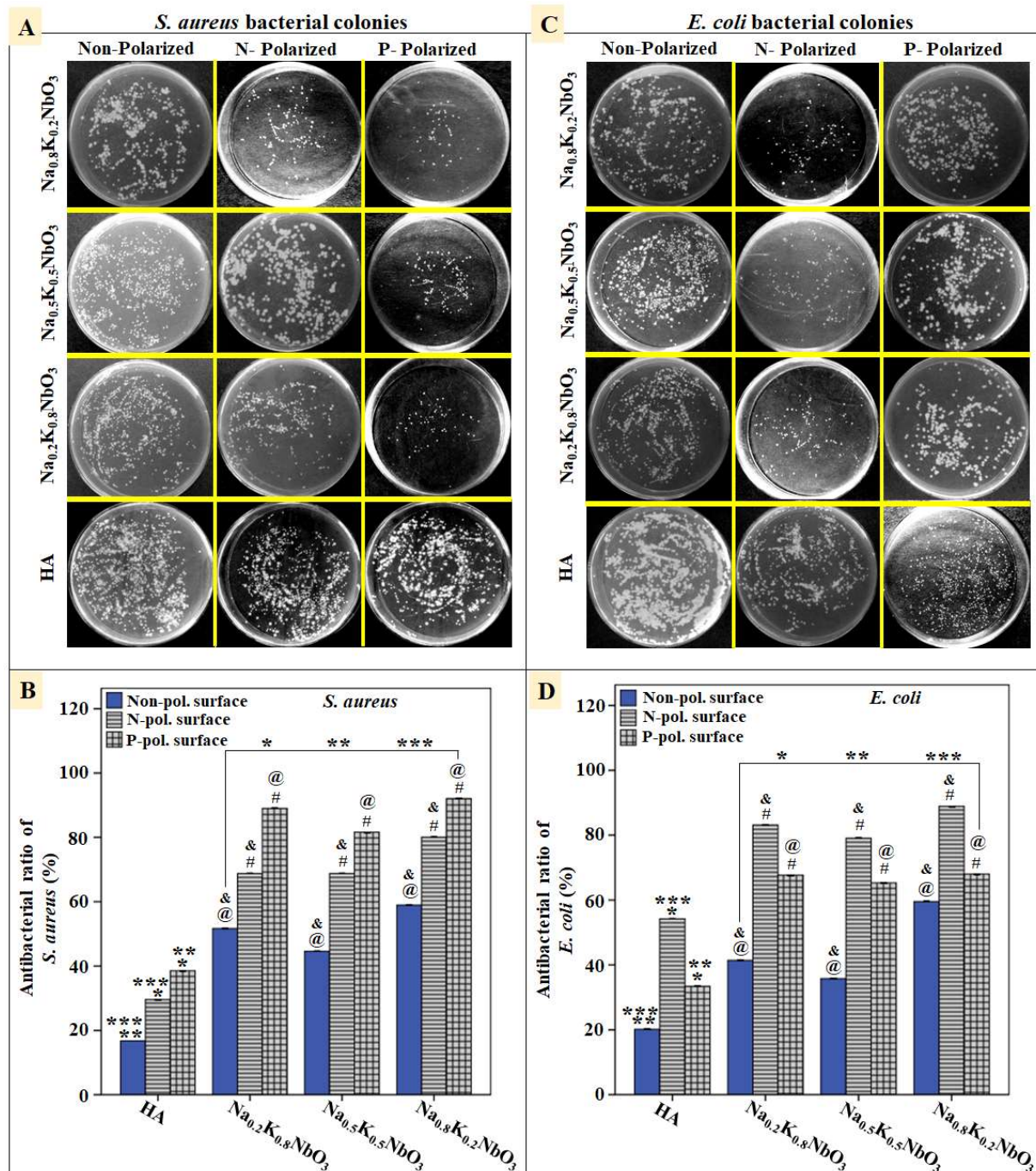
Fig. 5.4. Digital camera images of (A) S. aureus and (C) E. coli bacterial colonies on agar plate exposed with non-polarized and polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) and HA samples. The antibacterial ratio (calculated from eqn. 3.6, chapter 3) of (B) S. aureus and (D) E. coli bacteria cultured on non-polarized and polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) and HA

samples. Asterisk (*), (**) and (***) mark represent the statistically significant difference at $p \leq 0.05$ in the mean value of antibacterial ratio among all the samples with respect to non-polarized, negatively polarized and positively polarized HA, respectively. The symbols (#), (@) and (&) represent the significant difference at $p \leq 0.05$ in the mean value of antibacterial ratio among all the $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples with respect to their corresponding non-polarized, negatively polarized and positively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples.

5.3. Enzymatic activity for reactive oxygen species (ROS) measurement

It has been reported that the polarized surfaces are susceptible for the formation of reactive oxygen species (ROS), which make the surface bactericidal [1, 2, 3]. ROS such as, superoxides (O_2^-), peroxide (H_2O_2) etc. increases the level of oxidative stress and results in the rupture of bacterial cell membrane and consequently, causes bacterial cell death [2, 3]. In this perspective, different enzymatic assays such as superoxide dismutase (SOD), lipid peroxidation (LPO) and protein estimation assays have been performed to determine the amount of these free radicals, oxidative stress and protein damage, respectively. Primarily, the samples were seeded with *E. coli* and *S. aureus* bacteria and incubated for 8 h. Following this, culture from each sample was replaced by lysozyme (1 mg/ml) and further incubated for 1 h. This lysozyme added solution was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant (bacterial tissue homogenate) was used for the estimation of all enzymatic activities.

5.3.1. SOD assay

The SOD assay is generally performed to calculate the amount of antioxidant superoxide enzymes, produced on non-polarized and polarized samples of $Na_xK_{1-x}NbO_3$ and HA, while

cultured with *E. coli* and *S. aureus* bacteria. The bacterial tissue homogenate (500 μl) of each samples was taken in a test tube and 0.01 M PBS (pH ~ 7.8) was added. Following this, 130 mM methionine, 0.5 mM EDTA (Ethylenediamine tetracetic acid) and 0.75 mM NBT (Nitro blue tetrazolium) were added in SOD buffer. At the end, 60 μM riboflavin (in double distilled water) was added to complete the reaction mixture. The final solution was thoroughly mixed by shaking and kept in fluorescence light for 6 min. The absorbance of all the samples was taken at 560 nm in UV visible spectrophotometer, which is equivalent to the superoxide (O_2^-) production [4].

The effect of polarization on the production of superoxide on $Na_xK_{1-x}NbO_3$ samples for *S. aureus* and *E. coli* bacteria are demonstrated in Fig. 5.5 (a) and (b), respectively. The concentration of superoxide ions ($\bullet O_2^-$) are observed to be significantly higher on the positively polarized surfaces of $Na_xK_{1-x}NbO_3$ and control (HA) samples as compared to non-polarized and negatively polarized surface of respective samples, for both the bacterial cells. The superoxide ions production for *S. aureus* cultured surfaces of positively, negatively and non-polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples are (198, 185, 216 % of non-pol. HA), (141, 136, 143 % of non-pol. HA) and (118, 114, 119 % of non-pol. HA), respectively. For *E. coli* cultured surfaces, the superoxide ions production on the positively, negatively and non-polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples are (231, 223, 254 % of non-pol. HA), (133, 127, 136 % of non-pol. HA) and (116, 116, 129 % of non-pol. HA), respectively. It has been reported that surface polarization induces microelectric potential which further promotes the generation of ROS such as superoxide ions ($\bullet O_2^-$), preferably on the positively charged surface [5]. These superoxide ions converted into more reactive oxygen species

(such as hydroxyl) through Haber Weiss cycle reaction, which can directly damage the internal cellular component and deregulate the bacterial cells metabolism [6, 7].
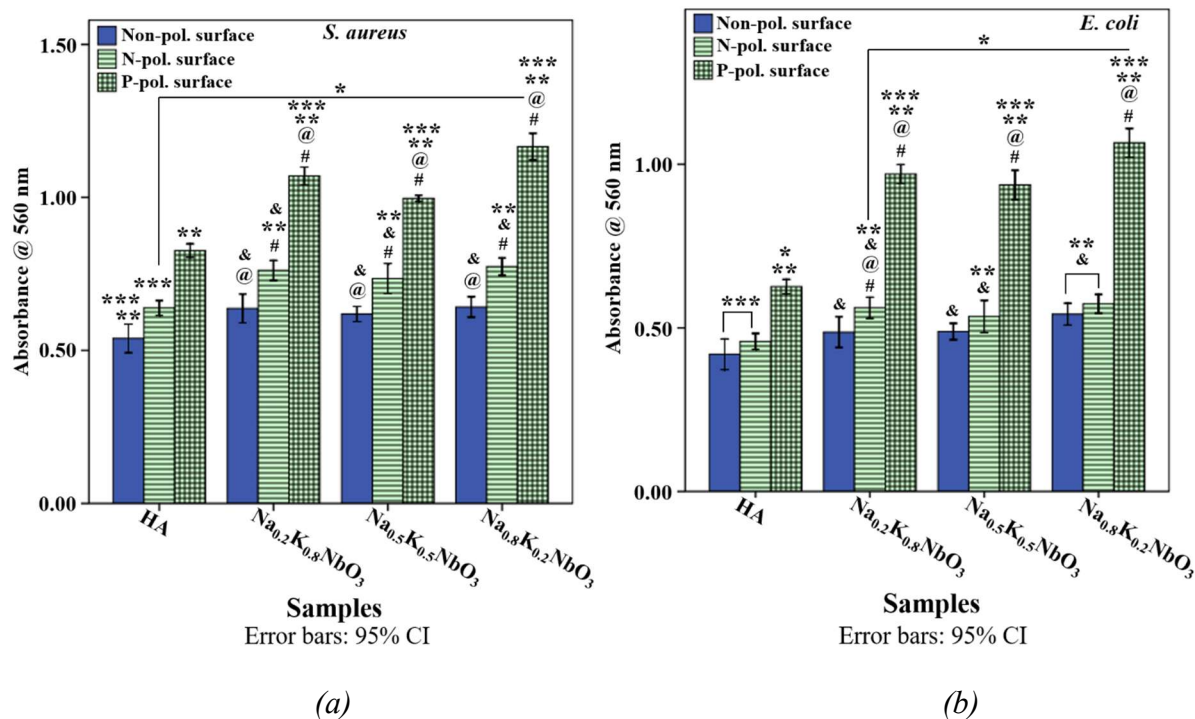

(a)   *(b)*

Fig. 5.5. The plots illustrating the concentration of superoxide ($\cdot O_2^-$) on non-polarized and polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) as well as HA samples for (a) S. aureus and (b) E. coli bacteria. Asterisk (*), (**) and (***) mark represents the statistically significant difference at p ≤ 0.05 in the mean absorbance among all the samples with respect to non-polarized, negatively polarized and positively polarized HA, respectively. Asterisk (#), (@) and (&) mark represent the significant difference at p ≤ 0.05 in the mean absorbance among all the $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples with respect to their corresponding non-polarized, negatively polarized and positively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples.

5.3.2. Catalase assay

Catalase activity was observed according to the Aebi method of catalase estimation, which quantify the dissociation of hydrogen peroxide (H_2O_2) radicals on non-polarized and polarized $Na_xK_{1-x}NbO_3$ as well as HA samples, while cultured with *E. coli* and *S. aureus* bacteria [8]. For this assay, the reaction mixture was prepared by taking 100 µl of bacterial tissue homogenate added with 50 mM phosphate buffer (pH = 7) and freshly prepared 30 mM H_2O_2. The absorbance of final reaction mixture was taken at the wavelength of 240 nm at interval of every 30 seconds upto 3 min. The dissociation of H_2O_2 was calculated in terms of catalase activity/s (K) as [8],

$$\text{Catalase activity (K)} = \frac{2.3}{\Delta t} \times \log \frac{E_1}{E_2} \qquad (5.1)$$

Where, E_1 and E_2 are the absorbance (concentration of H_2O_2), at $t = 0$ s and $t = 30$ s, respectively.

Fig. 5.6 (a) and (b) illustrates the effect of polarized surfaces of $Na_xK_{1-x}NbO_3$ samples on the catalase activity per second, against *S. aureus* and *E. coli* bacteria (eqn. 5.1). Here, catalase activity indicates time dependent dissociation of H_2O_2. It is observed that non-polarized, negatively and positively polarized surfaces of $Na_xK_{1-x}NbO_3$ samples show significant reduction in catalase activity as compared to non-polarized, negatively and positively polarized HA surfaces, respectively [Fig. 5.6 (a), (b)]. Statistical analyses also reveal that positively polarized surfaces of $Na_xK_{1-x}NbO_3$ as well as HA show significant depletion in catalase activity as compared to their respective non-polarized and negatively polarized surfaces [Fig. 5.6 (a), (b)]. The positively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) show acute depletion in catalase activity by (64, 65, 74 % of non-pol. HA) for *S. aureus* bacteria. However, it was just (29, 33, 39 % of non-pol. HA) and (12, 9, 6 % of non-pol. HA)

for negatively and non-polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples, respectively, while cultured with similar bacteria. Similarly, the positively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8), cultured with *E. coli* bacteria, show sharp decrease in catalase activity by (73, 66, 78 % of non-pol. HA). However, it was comparatively lower i.e., (48, 46, 43 % of non-pol. HA) and (20, 14, 17 % of non-pol. HA) on the negatively and non-polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples, respectively. It has been reported that reduced catalase activity is associated with slower dissociation of H_2O_2 and subsequent survival of reactive H_2O_2 which is toxic for bacterial cells [9, 10]. However, increased catalase activity indicates faster dissociation of H_2O_2 and subsequent increase in resistance of bacteria towards H_2O_2 [11].
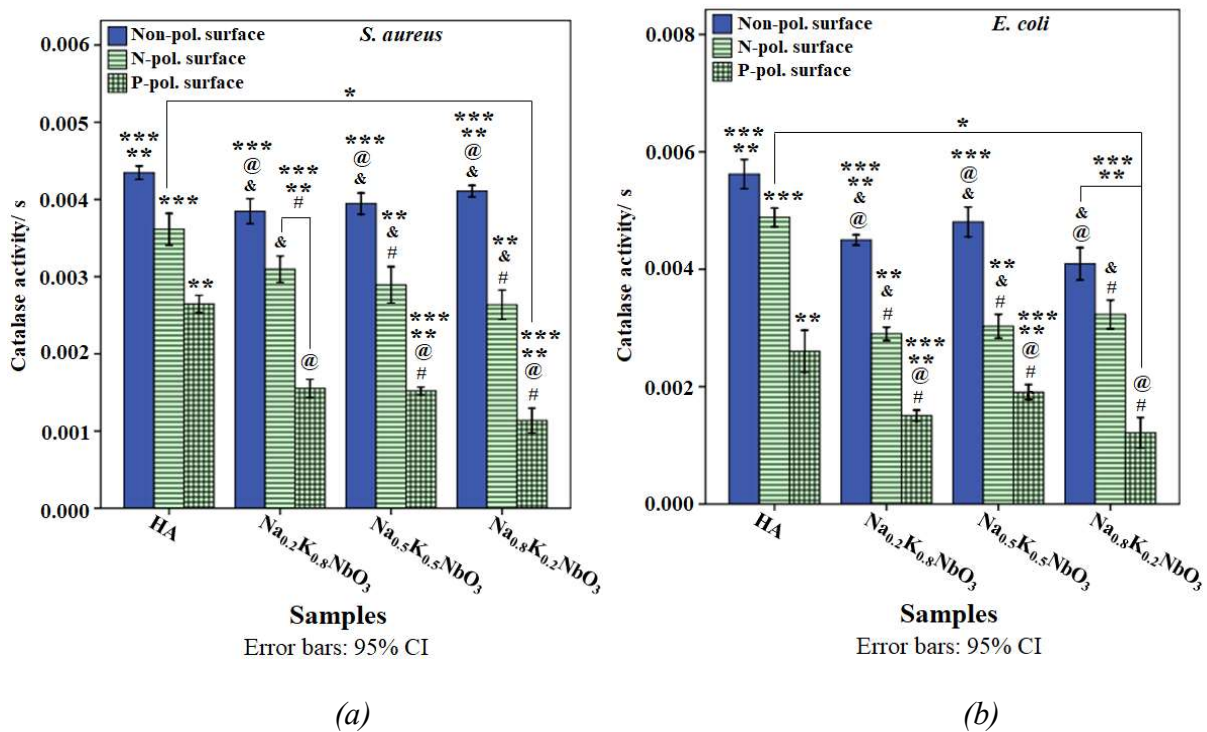

(a)   *(b)*

Fig. 5.6. The plots representing the catalase activity/ seconds (calculated from eqn. 5.1) on non-polarized and polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) as well as HA samples for (a) S. aureus and (b) E. coli bacteria. Asterisk (*), (**) and (***) mark represents the statistically

significant difference at p ≤ 0.05 in the mean value of catalase activity/ s absorbance among all the samples with respect to non-polarized, negatively polarized and positively polarized HA, respectively. The symbols (#), (@) and (&) represent the significant difference at p ≤ 0.05 in the mean value of catalase activity/ s among all the $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples with respect to their corresponding non-polarized, negatively polarized and positively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples.

5.3.3. ROS induced damaged protein estimation

The amount of oxidative stress induced protein leakage on non-polarized and polarized $Na_xK_{1-x}NbO_3$ as well as HA samples were determined by Lowery method using bovine serum albumin (BSA) as a standard, while the samples were cultured with *S. aureus* and *E. coli* bacterial cells [12]. In this assay, two reagents, reagent A (2 % Na_2CO_3 in 0.1 N NaOH) and reagent B (0.5 % $CuSO_4$ in 1.35 % potassium sodium tartrate) were used. Reagent C was prepared by the mixing of reagent A and B (24:1). In Lowery method, 20 µl of bacterial tissue homogenate of each sample was added with 980 µl of double distilled water in a test tube, followed by the addition of 5 ml of reagent C in each test tube and the solution was kept for 10 min. After that, 500 µl of folin's reagent (50 % in double distilled water) was added in each sample and again kept for 30 min in the dark. After 30 min, the color of the solution was turned to blue and absorbance of the samples was measured at 750 nm. The amount of oxidized protein (n mol./mg) on all the samples was calculated by Lowery method using BSA as a standard [12].

The comparative assessment of cellular protein leakage for *S. aureus* and *E. coli* bacteria, respectively, on non-polarized and polarized $Na_xK_{1-x}NbO_3$ and HA samples is illustrated as Fig. 5.7 Oxidative stress disrupts bacterial cell membranes and damages intracellular

component (DNA, protein etc.) which consequently, causes cell death [13]. In this assay, the amount of damaged protein, leaked from disrupted membranes has been measured. The amount of *S. aureus* bacterial protein leakage (n mol./mg) from positively, negatively and non-polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples are (38, 33, 41), (28, 25, 30) and (25, 20, 26), respectively. However, it was 28, 19 and 14 on the surfaces of positively, negatively and non-polarized HA samples, respectively. For *E.coli* bacteria, the amount (n mol./mg) of damaged protein from the positively, negatively and non-polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples are (42, 40, 46), (31, 29, 32) and (27, 25, 27), respectively. However, it was 31, 20 and 18 on the surfaces of positively, negatively and non-polarized HA samples, respectively. Statistical analyses reveal that the damaged bacterial protein leakage is significantly higher on non-polarized, negatively and positively polarized surfaces of $Na_xK_{1-x}NbO_3$ samples as compared to non-polarized, negatively and positively polarized HA surfaces, respectively, for both the bacteria [Fig. 5.7 (a), (b)]. In addition, positively polarized surfaces of $Na_xK_{1-x}NbO_3$ as well as HA show significant increase in damaged protein concentration as compared to their respective non-polarized and negatively polarized surfaces [Fig. 5.7 (a), (b)]. The positively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) show highest damaged protein leakage by (180, 140, 200 % of non-pol. HA) and (139, 129, 158 % of non-pol. HA) for *S. aureus* and *E. coli* bacteria, respectively. This assay further confirms the ROS induced bacterial damage on the positively polarized surfaces of $Na_xK_{1-x}NbO_3$ samples, which is preferably higher on the sodium and potassium rich phases of NKN i.e., $Na_{0.8}K_{0.2}NbO_3$ and $Na_{0.2}K_{0.8}NbO_3$ samples.
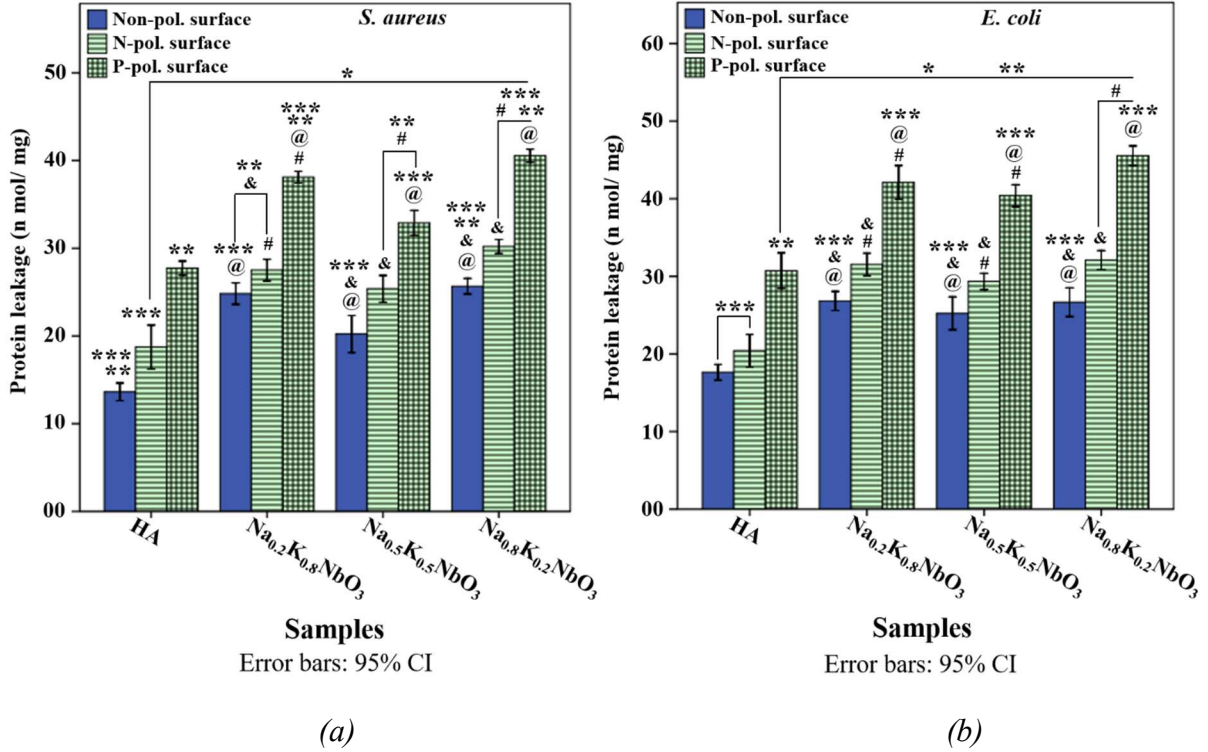
Fig. 5.7. The plots representing the leaked amount (n mol/ mg), of intracellular bacterial protein for (a) S. aureus and (b) E. coli bacteria on non-polarized and polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) and HA samples. Asterisk (*), (**) and (***) mark represents the statistically significant difference at $p \leq 0.05$ in the mean value of protein leakage (n mol/ mg) absorbance among all the samples with respect to non-polarized, negatively polarized and positively polarized HA, respectively. The symbols (#), (@) and (&) represent the significant difference at $p \leq 0.05$ in the mean value of protein leakage (n mol/ mg) among all the $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples with respect to their corresponding non-polarized, negatively polarized and positively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples.

5.3.4. LPO assay

LPO assay was performed to analyze the oxidative damage of *S. aureus* and *E. coli* bacterial cells on non-polarized and polarized $Na_xK_{1-x}NbO_3$ and HA samples. Lipid peroxidation is the indicator of oxidative damage of lipids and membrane of bacterial cells by the activation of

ROS [14, 15]. In this reaction, initially the peroxidation of unsaturated fatty acids takes place under the influence of ROS free radicals (singlet oxygen, hydroxyl radicals etc.) which forms unstable intermediate lipid hydroperoxides and hydroxyl radicals [16]. These reactive hydroxyl radicals further propagate the reaction and produce malondialdehyde (MDA), pentene, ethane, diens etc. as end product [15]. MDA cross-linked with DNA and proteins of bacterial cells and upregulated their expression which consequently, causes bacterial cell death. Therefore, ROS induced damage can be measured in terms of MDA [15]. For this assay, 500 µl of bacterial tissue homogenate was added with 500 µl of 0.1 M tris HCl solution (pH = 7.4) in a test tube and incubated at 37 °C for 2 h. After that, 1 ml of trichloroacetic acid (10 wt. % in double distilled water) was added and the solution was centrifuged at 3000 rpm for 10 min at 4 °C. Now, the obtained supernatant was mixed with equal amount of thiobarbituric acid. This mixture was boiled at 100 °C for 10 min and the color of the mixture was turned to red. The final solution was added with 1 ml of double distilled water before taking the absorbance at 532 nm. The LPO activities of *E. coli* and *S. aureus* bacteria were measured in terms of MDA, which can be calculated as [17],

$$\text{MDA/mg protein} = \frac{\text{OD (532)} \times \text{reaction Volume} \times 10^9}{\text{Sample volume} \times 1000 \times \text{Extinsion coefficient of MDA}} \quad (5.2)$$

Where, extinsion coefficient of MDA was taken as 1.56×10^5 M^{-1}cm^{-1}

Fig. 5.8 represents the comparative plots for produced oxidative stress (in terms of MDA concentration) on non-polarized and polarized $Na_xK_{1-x}NbO_3$ and HA samples. It has been already demonstrated that polarization induced microelectric field facilitates the formation of oxidative stress which can be measured as end product (MDA concentration) of LPO assay. Statistical analyses reveal that non-polarized, negatively and positively polarized surfaces of $Na_xK_{1-x}NbO_3$ samples show significantly higher MDA concentration as compared to non-

polarized, negatively and positively polarized HA surfaces, respectively [Fig. 5.8 (a), (b)] for

S. aureus as well as *E. coli* bacteria. The MDA concentration (n mol./mg), measured on the

S. aureus cultured non-polarized, negatively and positively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples are (0.98, 0.86, 1.02), (1.35, 1.34, 1.66) and (3.56, 2.66, 4.49), respectively, whereas, it was 0.72, 1.19 and 1.73 on the surfaces of non-polarized, negatively and positively polarized HA samples, respectively. The MDA concentration (n mol./mg) on the *E. coli* cultured non-polarized, negatively and positively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples are (2.37, 2.02, 3.73), (3.83, 3.33, 4.24) and (6.73, 5.37, 9.11), respectively, which was just 1.58, 2.72 and 3.96 on the surfaces of non-polarized, negatively and positively polarized HA samples, respectively. For *S. aureus* bacteria, positively and negatively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples show increased MDA concentration of about (4.9, 3.7, 6.2 times of non-pol. HA) and (1.9, 1.8, 2.3 times of non-pol. HA), respectively. Similarly, for *E. coli* bacteria, MDA level is observed to be (4.2, 3.4, 5.8 times of non-pol. HA) and (2.4, 2.1, 2.7 times of non-pol. HA), for positively and negatively polarized surfaces of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples, respectively.

The enzymatic analyses on the non-polarized and polarized NKN samples reveal that ROS activity and ROS induced bacterial damage is maximum on the positively polarized NKN samples, preferably on the sodium and potassium rich NKN i.e., $Na_{0.8}K_{0.2}NbO_3$ and $Na_{0.2}K_{0.8}NbO_3$ samples. Therefore, these analyses further corroborate the results obtained from Live/ dead assay [Fig. 5.3].

ROS generation from polarized surfaces promotes the killing of bacteria. However, ROS species activate mitogen-activated protein kinases (MAPK), which promote the growth of

osteoblast like cells [18, 19]. Therefore, ROS generation on the polarized surfaces, positively influences the growth of bone-related cells.
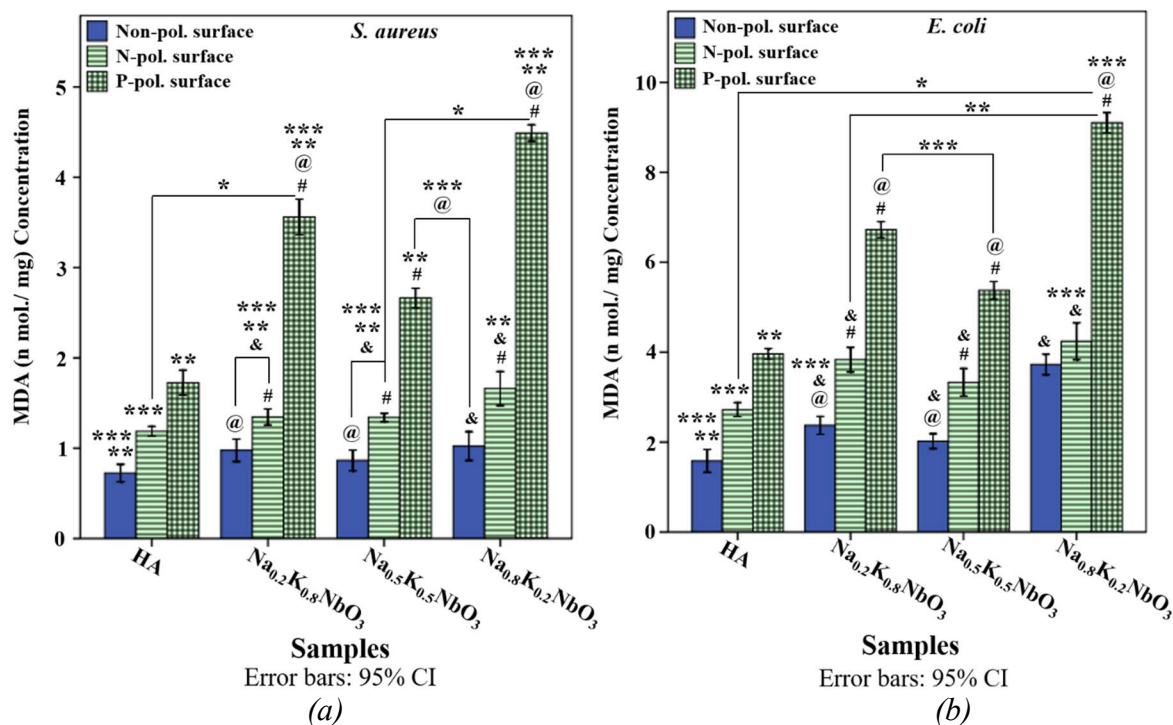

Fig. 5.8. The plots representing the MDA concentration (nmol./mg) (calculated from eqn. 5.2) for (a) S. aureus and (b) E. coli bacteria, cultured on non-polarized and polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) and HA samples. Absorbance were measured at 532 nm and MDA concentration were calculated in n mol. /mg of protein (from eqn. 5.2). Asterisk (*), (**) and (***) mark represents the statistically significant difference at p $\leq$ 0.05 in the mean value of MDA concentration production among all the samples with respect to non-polarized, negatively polarized and positively polarized HA, respectively. The symbols (#), (@) and (&) represent the significant difference at p $\leq$ 0.05 in the mean value of MDA concentration produced among all the $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples with respect to their corresponding non-polarized, negatively polarized and positively polarized $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) samples.

In view of above mentioned results, it can be revealed that the antibacterial response of piezoelectric $Na_xK_{1-x}NbO_3$ is sensitive to the surface charge polarity, type of bacteria and compositional variation in sodium and potassium. The present study shows that the positively polarized surfaces of $Na_xK_{1-x}NbO_3$ ceramics result in increased production of ROS such as superoxides [Fig. 5.5] and permits significantly higher lipid peroxidation [Fig. 5.8] as compared to their corresponding negatively polarized and non-polarized surfaces as well as polarized and non-polarized HA. The catalase activity [Fig. 5.6] is also comparatively reduced on the positively polarized surfaces of $Na_xK_{1-x}NbO_3$ samples than their negatively polarized and non-polarized surfaces as well as non-polarized/ polarized surfaces of control, which indicate the presence of larger amount of ROS (peroxide) on the positively polarized surfaces of $Na_xK_{1-x}NbO_3$ samples. Fig. 5.7 indicates that the bacterial protein leakage is also significantly higher on the positively polarized surfaces of $Na_xK_{1-x}NbO_3$ samples than their respective negatively polarized and non-polarized counterparts as well as non-polarized/ polarized control, which reveal that the positively polarized surfaces lead to the larger amount of cytoplasmic component damage of the bacterial cells [Fig. 5.9]. In addition, the positively polarized surfaces depolarize the negatively charged membrane of the bacterial cells [20]. It changes the permeability of cellular membrane and results in the leakage of intracellular components (e.g., glucose, proteins, nucleic acid etc.), and causes cell death [Fig. 5.9] [20]. Therefore, the findings of enzymatic activities supports the qualitative assessment of bacterial adhesion that the positively polarized $Na_xK_{1-x}NbO_3$ ceramic surfaces show comparatively higher amount of dead bacteria than negatively polarized and non-polarized NKN samples as well as non-polarized/ polarized control [Fig. 5.3]. The negatively charged surfaces of $Na_xK_{1-x}NbO_3$ ceramics also inhibit the adhesion of bacteria due to

electrostatic repulsion [Fig. 5.9] [20]. Therefore, live as well as dead bacterial cells are observed to be reduced on the negatively charged surfaces of $Na_xK_{1-x}NbO_3$ ceramics however, positively polarized surfaces show larger amount of dead bacterial cells as compared to the negatively polarized as well as non-polarized surfaces [Fig. 5.3]. Apart from electrostatic repulsion, hydrophilicity is another factor which improves the antibacterial response of negatively polarized surfaces [21, 22, 23, 24, 25].

It has been reported that small sized particles enter in the nanosized pores of bacteria and disturb cell metabolism which consequently, results in growth inhibition or death of bacterial cells [Fig. 5.9] [26, 27]. In $Na_xK_{1-x}NbO_3$ ceramics, the smaller sized sodium more readily interact with pores of bacteria than potassium having larger size than sodium [28]. It may be one of the reasons for comparatively higher antibacterial response of $Na_{0.8}K_{0.2}NbO_3$ (sodium rich phase) than $Na_{0.2}K_{0.8}NbO_3$ (potassium rich phase) and $Na_{0.5}K_{0.5}NbO_3$ samples [Figs. 5.1, 5.2, 5.3 and 5.4]. Another reason for excellent bacterial inhibition of sodium rich phase of NKN i.e., $Na_{0.8}K_{0.2}NbO_3$ is that the higher concentration of sodium ion (with lower concentration of potassium ion) changes the extracellular osmotic condition which restricts the growth of microorganisms [29]. Potassium has been reported to be more hygroscopic than that of sodium [30]. The hygroscopic surfaces, when comes in contact with bacteria, absorbs intracellular water through the cell wall at the interface and consequently causes bacterial growth inhibition [31, 32]. It may be one of the reasons that potassium rich phases of NKN ($Na_{0.2}K_{0.8}NbO_3$) show good antibacterial response. The results obtained from the enzymatic activities also indicate that sodium and potassium rich compositions of NKN generate a significant amount of ROS which makes it antibacterial. Overall, it can be suggested that the antibacterial response of polarized $Na_xK_{1-x}NbO_3$ ceramics can be

significantly improved by harmonic compositional variation of sodium and potassium in NKN.
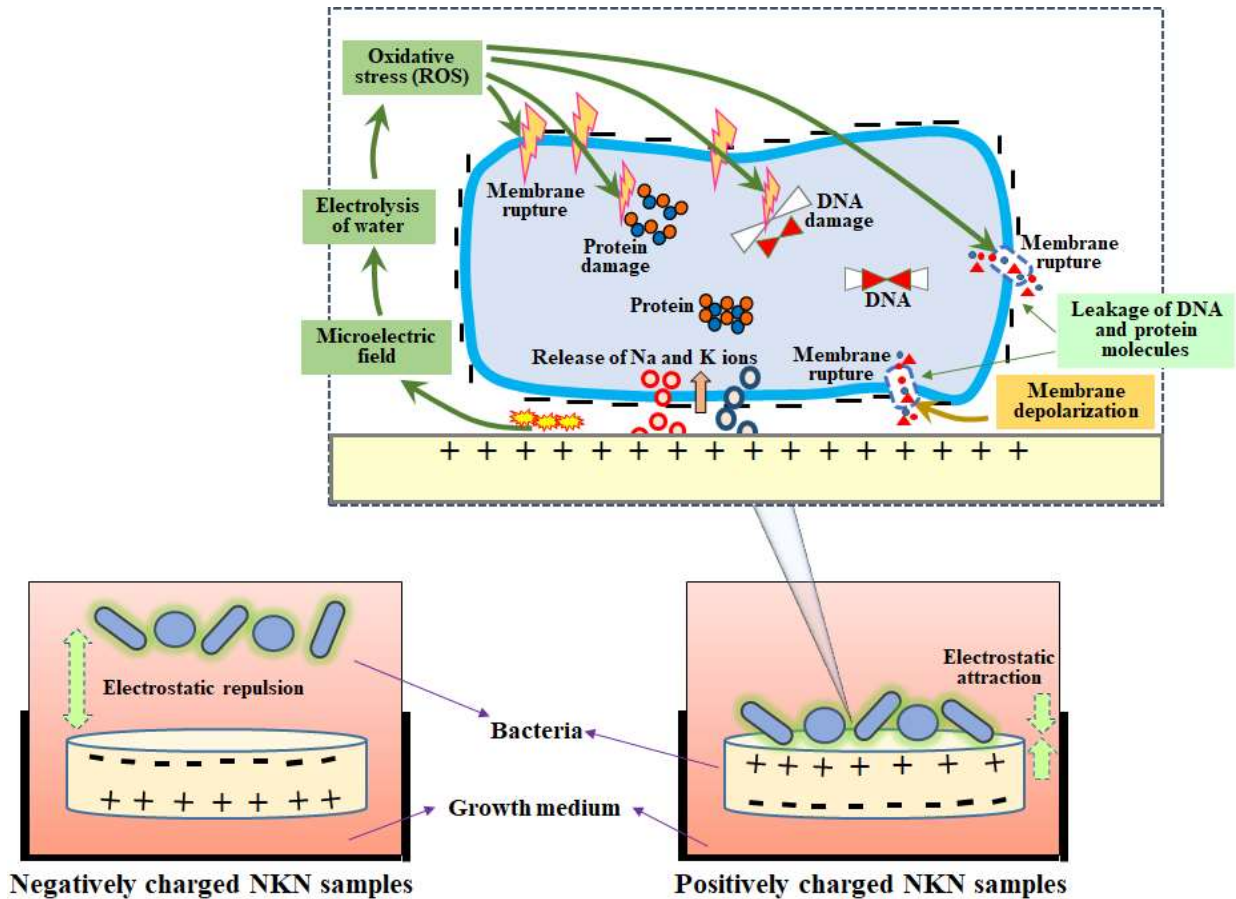

Fig. 5.9. Schematic representing various mechanisms of antibacterial response of NKN samples, induced by surface charge polarization [1, 3, 4, 13, 15, 20, 29, 33].

In the present work, the processing conditions for the development of dense and phase pure $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8) have been successfully optimized. X-ray diffraction patterns and FTIR spectra confirm the formation of phase pure $Na_xK_{1-x}NbO_3$ (x = 0.2 - 0.8). Lattice parameters were also calculated using Rietveld refinement. The average particle size of $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) powder samples, obtained from scanning electron microscopy (SEM) are 630 ± 95 nm, 580 ± 75 nm and 470 ± 70 nm, respectively. MTT and disk diffusion results demonstrated that the non-polarized NKN samples show significant

reduction in bacterial adhesion as compared to non-polarized HA which was further reduced by surface polarization. The viability of *S. aureus* bacteria on non-polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) ceramic samples has been reduced by (18, 10, 15 %) as compared to non-polarized HA which has further reduced by (41, 29, 50 %) with positive polarization and by (28, 20, 30 %) with negative charge. Similarly, for *E. coli* bacteria, the viability has been reduced by (20, 17, 22 %) on the non-polarized $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) samples which has further reduced by (49, 37, 52 %) with negative charge and (31, 24, 44 %) with positive charge polarizations as compared to non-polarized HA. The polarized surfaces of sodium rich (x = 0.8) and potassium rich (x = 0.2) phases of NKN are observed to inhibit the bacterial growth. Overall, the present study reveals that polarized and compositionally tailored $Na_xK_{1-x}NbO_3$ (x = 0.2, 0.5, 0.8) ceramics exhibit excellent antibacterial response and could be a potential candidate for prosthetic orthopedic implant.

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